

# Stem Cell Emergence and Hemopoietic Activity Are Incompatible in Mouse Intraembryonic Sites

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## Summary

In the mouse embryo, the generation of candidate progenitors for long-lasting hemopoiesis has been reported in the paraaortic splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) region. Here, we address the following question: can the P-Sp/AGM environment support hemopoietic differentiation as well as generate stem cells, and, conversely, are other sites where hemopoietic differentiation occurs capable of generating stem cells? Although P-Sp/AGM generates *de novo* hemopoietic stem cells between 9.5 and 12.5 days post coitus (dpc), we show here that it does not support hemopoietic differentiation. Among mesoderm-derived sites, spleen and omentum were shown to be colonized by exogenous cells in the same fashion as the fetal liver. Cells colonizing the spleen were multipotent and pursued their evolution to committed progenitors in this organ. In contrast, the omentum, which was colonized by lymphoid-committed progenitors that did not expand, cannot be considered as a hemopoietic organ. From these data, stem cell generation appears incompatible with hemopoietic activity. At the peak of hemopoietic progenitor production in the P-Sp/AGM, between 10.5 and 11.5 dpc, multipotent cells were found at the exceptional frequency of 1 out of 12 total cells and 1 out of 4 AA4.1<sup>+</sup> cells. Thus, progenitors within this region constitute a pool of undifferentiated hemopoietic cells readily accessible for characterization.

Key words: aorta-gonad-mesonephros • spleen • omentum • hemopoiesis • reconstitution

During ontogeny, it is intriguing that apart from the yolk sac (YS),<sup>1</sup> which both produces hemopoietic progenitors and carries out erythropoiesis, other organs where active hemopoiesis takes place, such as the thymus and fetal liver, must be colonized by extrinsic hemopoietic stem cells (HSCs) or progenitors (1–3). Here we attempted to understand the constraints affecting these processes by establishing how far progenitors generated in the paraaortic splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) region could evolve *in situ*, and by determining whether the spleen and omentum, i.e., mesodermal structures derived from the P-Sp, have a similar or different capacity in this regard.

We have previously demonstrated that, before circulation is established, progenitors emerging in the intraembry-

onic splanchnic mesoderm are capable of generating all hemopoietic lineages (4). In contrast, YS progenitors have a restricted proliferative capacity and yield erythromyeloid cells (4). Altogether, the results indicate that the colonizing multipotent cells come from the embryo proper, from the region comprising the aorta and the mesodermal territory where the genital ridges and mesonephros later develop. It has been called P-Sp at the stages of 15–25S (8.5–9.5 days post coitus [dpc]; reference 5) and AGM thereafter (6, 7). After the establishment of blood circulation (5–8S, 8 dpc), multipotent cells can be found in the blood vessels and colonize the hemopoietic rudiments (5–8).

Here we analyzed the differentiation potential of hemopoietic cells in the P-Sp/AGM. We failed to detect progenitors committed to specific lineages, as ascertained by single cell analysis and by the absence of committed erythroid colony-forming precursors. Thus, cells within this region remain essentially multipotent. The quantification of progenitors by limiting dilution assays showed a continuous production starting by 9 dpc in the P-Sp and becoming

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<sup>1</sup>Abbreviations used in this paper: AGM, aorta-gonad-mesonephros; BFU-E, burst-forming unit-erythroid; CFC, colony-forming cell; CFU-S, colony-forming unit-splenic; dpc, day(s) post coitus; Epo, erythropoietin; FTOC, fetal thymic organ culture; HSC, hemopoietic stem cell; KL, c-Kit ligand; LTR, long-term reconstitution; PeC, peritoneal cavity; P-Sp, paraaortic splanchnopleura; YS, yolk sac.

completely extinguished by 13 dpc in the AGM, with a peak at ~11 dpc. Finally, within the AGM, hemopoietic progenitors were found concentrated in the aorta and surrounding mesenchymal cells.

There is a paradox in the embryogenesis of the hemopoietic system which is as follows: contact with endoderm appears necessary for the emergence of hemopoietic progenitors (9–11), yet hemopoietic organ rudiments that are constituted of mesoderm and endoderm, such as the thymus and liver, do not give rise to hemopoietic progenitors, thus requiring colonization by extrinsic progenitors. The omentum and spleen are associated with the stomach mesoderm and endoderm. Actually, omentum and spleen anlage belong primitively to the P-Sp, raising the possibility that hemopoiesis detected in these organs derives from *in situ*-generated progenitors. The extrinsic origin of splenic hemopoietic progenitors in the spleen has been documented in the chick embryo through parabiosis experiments (12) and transplantation assays (13). In the mouse, hemopoiesis in the splenic rudiment is also thought to result from colonization, but the evidence is descriptive (14). In the spleen, hemopoietic activity has been documented from 16–17 dpc (14) until about the first week of postnatal life. B cell precursors have been detected in the omentum by 14 dpc (15–17).

As no data are available about early hemopoiesis in the omentum, and information in the case of the spleen is scarce, we analyzed these two sites for their ability (or inability) to generate HSCs, on the one hand, and to sustain hemopoiesis, on the other. Both organs were unable to generate hemopoietic progenitors when separated from the embryo before 12 dpc, even if precultured before dissociation, a method that had permitted the detection of progenitor emergence in the presomitic embryonic compartment (4). We report that the spleen is colonized after day 12 of gestation by extrinsic multipotent progenitors, most likely AGM derived. The splenic environment sustains self-renewal of HSCs and differentiation of their progeny, as determined by the presence of long-term reconstituting (LTR) progenitors and by the increasing number of lymphoid and erythromyeloid colony-forming cells (CFCs) with the developmental stage. In contrast, after colonization at 14.5 dpc, the omentum environment is shown to provide for the survival of limited numbers of lymphocyte precursors. The absence of both erythromyeloid CFCs and LTR activity indicates that the omentum is not a hemopoietic organ. This study, together with previously reported results, indicates that all analyzed hemopoietic organs (fetal liver, thymus, spleen, and bone marrow) and the omentum are colonized tissues. In the murine model, P-Sp/AGM-derived HSCs might thus constitute the only pool of HSCs produced *de novo* within the embryo.

## Materials and Methods

### *Mice and Cell Preparations*

The following mouse strains were used in the course of this study. BALB/c mice provided embryos for precursor numeration. The differentiation potential analysis and the cell fractionation were performed using embryos from the two C57BL/6 congenic

lines bearing the Ly5.2 and Ly5.1 alleles of the panhemopoietic marker CD45. F1 embryos resulting from the cross of the two C57BL/6 lines were also used.

Mature females were caged with breeding males. The day of vaginal plug observation was considered as 0.5 dpc. Pregnant females between 9.5 and 15.5 dpc were killed by cervical dislocation. Embryos were staged by somite counting and/or by development of the limb buds. The various rudiments were dissected under a stereomicroscope, and single cell suspensions were prepared by passage of the selected tissues through a 26-gauge needle. Viable cells were counted by trypan blue exclusion.

### *Basic Culture Conditions*

The basic conditions were described previously (18, 19). Cells were cultured on the S17 stromal line (a gift from K. Dorshkind, University of California at Riverside, Riverside, CA) with the following cytokines: IL-7 at 50–100 U/ml was provided by the supernatant of a stably transfected cell line (from Fritz Melchers, Basel Institute for Immunology, Basel, Switzerland), and c-Kit ligand (KL; Genetics Institute) was used at a 1:500 dilution, which allows the emergence of mast cells from adult bone marrow.

Cells were seeded at limiting dilution or micromanipulated, then plated in 48–96 wells from 96-well plates for each cell concentration, in culture medium (OptiMEM [GIBCO BRL] plus 10% FCS) supplemented with IL-7 and KL. At day 10 of culture, cells from individual wells were harvested and divided into three fractions. Each fraction was transferred to culture conditions that promote erythromyeloid, lymphoid B, or lymphoid T differentiation.

**Erythromyeloid Conditions.** Cells were cultured on S17 stromal cells (known to produce GM- and M-CSF) in the presence of KL, human recombinant erythropoietin (Epo, 4 U/ml; a gift of E. Goldwasser, University of Chicago, Chicago, IL), GM-CSF at 4 ng/ml, and IL-3 (supernatant from a transfected cell line from F. Melchers), used at a 1:100 dilution. Cells retrieved from the culture were analyzed for Gr-1 and Ter-119 expression as well as by Giemsa staining.

**B Lymphoid Conditions.** Cells were cultured on S17 stromal cells with medium supplemented only with IL-7. For analysis of mature B cells, cells were stimulated with LPS (*Salmonella typhosa* W0901; Difco) as described previously (20), and Ig secretion was detected in an ELISA.

**T Lymphoid Conditions.** The third fraction was placed in fetal thymic organ cultures (FTOCs [21]) using recipient thymic lobes from 14–15-dpc Ly5 congenic C57BL/6 mice bearing a Ly5 allele differing from that of donor cells, as described previously (5). In brief, 30  $\mu$ l of the cell suspension was distributed between three and four irradiated fetal thymic lobes in wells of a Terasaki plate and cultivated in a hanging drop for 24–48 h. Colonized thymic lobes were cultured for 10–13 d on polycarbonate filters (0.8  $\mu$ m; Millipore) floating on top of the culture medium. To analyze cells from repopulated thymuses, single cell suspensions were made by teasing the organs with two needles. The cells obtained from three to four thymic lobes repopulated with cells from the same clone were pooled for flow cytometry analysis. To calculate the frequency of T cell precursors in the omentum, we colonized each of 10–15 irradiated thymic lobes with a constant number of cells. Three different cell concentrations were used. 12 d later, individual lobes were teased, and cells were analyzed by flow cytometry. The frequency of T cell precursors was then calculated by a Poisson distribution analysis.

### *Organotypic Culture*

Explanted tissues were placed directly on a polycarbonate filter as described above, except that  $5 \times 10^{-5}$  M  $\beta$ -ME was included

in the culture medium. After 10 d of culture, the explants were mechanically dissociated before flow cytometry analysis.

### *In Vitro Colony Assay*

AGM (10.5–11.5 dpc), omentum, and spleen (11–15.5 dpc) were dissected and dissociated.  $5 \times 10^3$  or  $5 \times 10^4$  cells from each sample were mixed with OptiMEM, 0.8% methylcellulose (15 mPAS; Fluka), and 10% FCS, supplemented with IL-11, KL, IL-3, GM-CSF, and Epo.

Colonies were scored at day 3 (CFU-E) and day 7 (burst-forming units-erythroid [BFU-E] and CFC-Mix [see below]). Colonies of well-hemoglobinized clusters of <100 cells were classified as CFU-E. Large colonies of red cells (>300 cells) were counted as BFU-E, while colonies containing at least 2 myeloid cell types and erythroid cells were classified as CFC-Mix.

### *In Vivo Reconstitution Experiments*

To test for LTR potential, cells from 13–14.5-dpc C57BL/6 embryo omentum and spleen were injected in the retroorbital sinus of lethally irradiated (800–850 rad) C57BL/6 mice bearing an Ly5 allele differing from donor embryos. The mice received in addition  $5 \times 10^5$  adult bone marrow cells bearing the same Ly5 allele as the recipient. Control mice were injected with PBS. After 6–8 mo, the recipient mice were killed; the bone marrow, spleen, thymus, and cells from the peritoneal cavity (PeC) were then collected and analyzed by flow cytometry.

### *Flow Cytometry Analysis*

Flow cytometry analysis was performed in a FACScan® with the CellQuest program (Becton Dickinson). The Ly5 alleles were characterized using biotinylated or fluorescein-conjugated antibodies purified from the supernatant of the 104.2 (anti-Ly5.2) or A20.17 (anti-Ly5.1) hybridoma lines. The following antibodies were used to label B and T lymphocytes: anti-CD45R/B220 (clone RA3-6B2), anti-CD4 (L3T4), all directly coupled to PE, anti-CD8 (Ly-2) coupled to FITC, and biotinylated CD5. PE-conjugated Gr-1 and biotinylated Ter-119 were used to characterize cells from the myeloid and erythroid lineages, respectively. All antibodies were from PharMingen. Streptavidin-Tricolor (Caltag) was used as a second step reagent. In all analyses, propidium iodide was used to exclude dead cells.

## **Results**

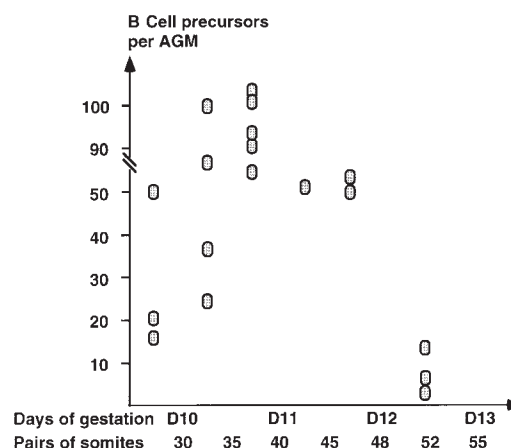
*Hemopoietic Progenitors Are Generated in the P-Sp/AGM from 8.5 to 12.5 dpc.* We have previously shown that multipotent hemopoietic progenitors are detected in the P-Sp region starting at the stage of 10 somites (8.5 dpc) and increase thereafter, reaching 15 detected progenitors per explant at the 25-somite stage (9.5–10 dpc [5]). This region has been called the AGM region after 9.5 dpc, and has been shown to harbor HSCs (6, 7). However, the numbers of generated hemopoietic cells in this region and the duration of the process have been important missing information. Here, we approached these questions by performing a stage by stage quantitative analysis of progenitors capable of generating B lymphocytes in the AGM region over a period of time extending from 9.5 to 13 dpc. Limiting dilution tests were done under in vitro conditions that support the generation of B cells from multipotent progenitors. Approximately 20 progenitors were detected per AGM at 9.5 dpc

(25 somites; Fig. 1). This number increased to a maximum of 100 between 10.5 and 11.5 dpc (35–45 somites), as fetal liver hemopoietic activity began. A large variation between embryos was observed between the 25- and 45-somite stages. Thereafter, progenitors dramatically decreased to a barely detectable number by 12.5 dpc.

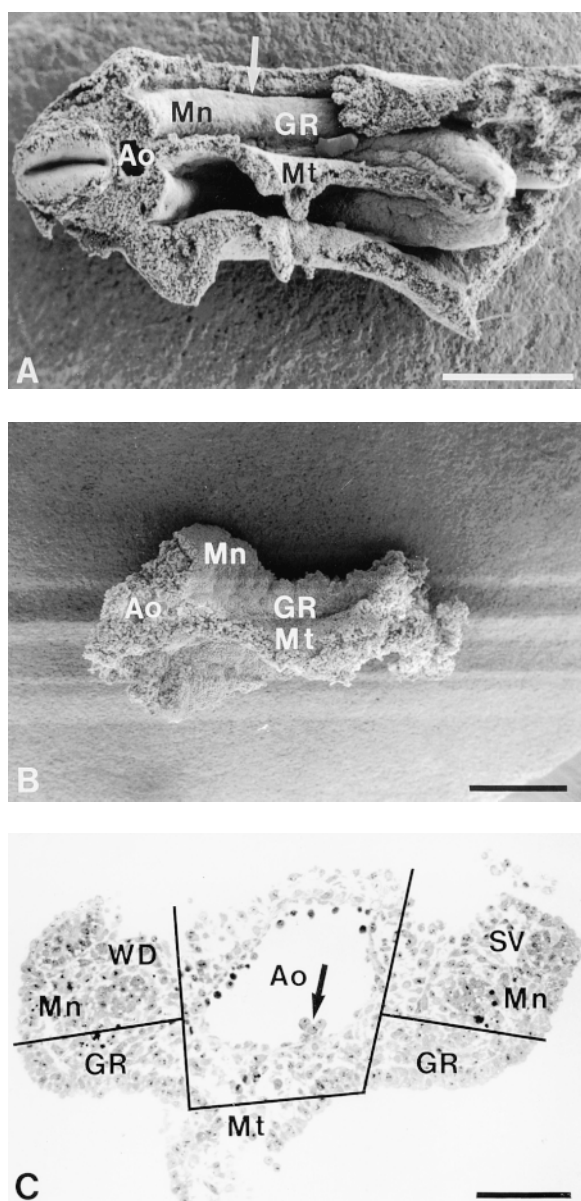
These experiments, with previous studies (5), indicate that the P-Sp/AGM region is continuously active as a site of hemopoietic cell generation from days 8.5–12.5 of mouse gestation. The maximum activity was detected between 10.5 and 11 dpc, coinciding with the period of fetal liver and thymus colonization. At 13 dpc, hemopoietic generation in the P-Sp/AGM region became extinguished.

*Within the AGM, Hemopoietic Progenitors Are Concentrated in the Aorta.* In an attempt to precisely determine the distribution of hemopoietic progenitors within the AGM, we further dissected this region into its various components (Fig. 2 A–C): the aortic endothelium and its wall, surrounded by mesenchymal cells; the mesonephros, including the Wolffian duct with adjacent mesonephretic cells; the genital ridges; and the mesentery lying ventrally to the aorta, from which the developing gut had been removed. B cell progenitors within each of these regions were again quantified.

Due to the minute amount of material in the various explants, cross-contamination between the different components of the AGM could not be avoided. Nevertheless, the results of two experiments (Table I) carried out at the 30–35- and 35–40-somite stages, when the precursor content in the AGM reaches a peak, indicate that the aorta is highly enriched in these progenitors. At this stage, and without any cell purification, the frequency of hemopoietic clones in the aorta was 1:12. At the time when the number of progenitors per AGM began to decrease, the aorta separated from the other components of the AGM gave rise to similar numbers of lymphocyte clones as the whole unseparated region (Fig. 1). The mesonephros repeatedly displayed the lowest number of progenitors. As for the gonads and the mesentery, the precursor content was more vari-



**Figure 1.** Evolution of absolute numbers of B cell progenitors detected per AGM over the midgestation period. Progenitors endowed with B lymphoid potential were quantified through limiting dilution analysis.



**Figure 2.** Structure and dissection of the AGM. (A) Scanning electron microscopy of a partially dissected AGM (bar, 0.22 mm). (B) Scanning electron microscopy of an explanted AGM. The anterior part of the explant (left) is considerably larger than the caudal part (bar, 1 mm). (C) Semithin section of an AGM explant. Note the hemopoietic cell cluster (reference 27) associated with the endothelium of the ventral part of the aorta (arrow; bar, 0.26 mm). Ao, aorta; GR, genital ridges; Mn, mesonephros; Mt, mesentery; SV, subcardinal vein; WD, Wolffian duct.

able, possibly depending on the degree of contamination by cells from the region underlying the aorta (Table I).

In a similar experiment performed at the 50–55-somite stage (12.5 dpc), when progenitors in the AGM were disappearing, all remaining progenitors were located in the aorta and the surrounding tissue (data not shown).

The distribution of progenitors along the antero-posterior axis was also tested in a similar way, in two independent experiments. The AGM explant was divided into two (Table I) or three sections (data not shown). Although the

**Table I.** Distribution of Hemopoietic Progenitors within the AGM

Tissue	30–35 somites		35–40 somites	
	Frequency	Precursors/organ	Frequency	Precursors/organ
Mesonephros	1/150	9 (9.89%)	1/1,000	<0.1 (0%)
Mesentery	1/90	28 (30.77%)	1/75	17 (20.48%)
Gonad	1/400	4 (4.39%)	1/16	14 (16.86%)
Aorta	1/35	50 (54.94%)	1/12	50 (62.65%)
Anterior	1/100	100		ND
Caudal	1/65	49		ND

AGM was further dissected into its various components or, alternatively, into anterior and posterior regions. After dissociation, precursor numbers in each component were evaluated through limiting dilution analysis.

total number of progenitors predominated in the anterior region located immediately below the fetal liver, their frequency was higher in the intermediate and caudal regions.

In conclusion, hemopoietic progenitors in the AGM were virtually absent from the most lateral components of the AGM (mesonephros and gonads), and more than half of these progenitors were concentrated in the aorta region.

**AGM Is Not a Site of Ongoing Hemopoiesis.** Although the environment of the P-Sp/AGM region is specifically generating hemopoietic cells de novo, it is unclear whether lineage commitment also occurs there. We analyzed the capacity of the progeny of micromanipulated individual cells to differentiate into erythromyeloid and lymphoid cells when seeded in culture conditions promoting colony formation from BFU-E, CFU-E, and CFC-Mix. We chose to enrich hemopoietic progenitors based on the expression of antigens defining populations comprising LTR HSCs, either in the fetal liver (AA4.1 [22]) or in adult bone marrow (Sca-1 [23]).

After a period of expansion, the progeny of individual cells previously enriched for AA4.1 expression was tested for the capacity to generate B or T lymphoid as well as erythromyeloid precursors (5; see also Materials and Methods). Table II shows the number of multipotent clones found at the various stages analyzed. These numbers mostly correlate with those of B cell precursors detected previously (compare Fig. 1 and Table II, top). Fig. 3 shows the flow cytometric profiles of the progeny of one representative micromanipulated precursor. After FTOC (top panel), single- and double-positive CD4/CD8 thymocytes expressing intermediate and high levels of TCR- $\alpha/\beta$  were present in the reconstituted lobes. B lineage cells developed in culture on stromal cells with IL-7 (bottom panel) and, further stimulated by LPS, were shown to secrete immunoglobulins. May-Grünwald Giemsa staining of precursors from the same progenitor expanded with KL, GM-CSF, IL-3, and Epo showed the development of multilineage myeloid cells (data not shown).

The experiment performed at the 50–55S stage (representing a pool of 24 AGMs) yielded 27 colonies, a value

**Table II.** Differentiation Potential of Single AA4.1<sup>+</sup> or Sca-1<sup>+</sup> Cells from the AGM or the Aorta

Stage (no. of somites)	Percent total cells	Cloning efficiency	Multipotent clones/organ
AGM			
30–35	0.3% AA4.1 <sup>+</sup>	1/6	24
35–45	ND	1/4	50
50–55	ND	1/21	27
Aorta			
48–52	3.25% AA4.1 <sup>+</sup>	1/4	65
48–52	2.5% Sca-1 <sup>+</sup>	1/33	6

Clones generated from individual micromanipulated cells were fractionated and further cultured in conditions promoting erythroid, myeloid, and lymphoid B and T lineages. Clones were considered multipotent when they gave rise to both lymphoid and myeloid derivatives. All clones that generated lymphocytes also gave rise to multiple myeloid progeny. The clones that only gave rise to single myeloid progeny (typically macrophages) were not taken into account. The ratio between growing clones and the number of cells plated defines cloning efficiency. In all experiments, cells were plated at 0.5 cell per well or micromanipulated.

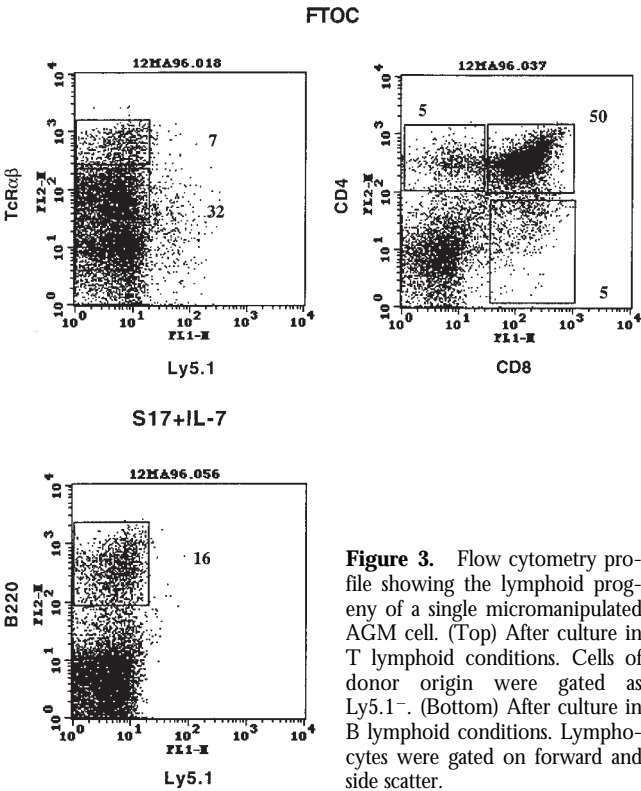
larger than that obtained during precursor numeration through limiting dilution (5–20; Fig. 1 and Table II). This discrepancy may either reflect individual variations or may be due to the use of a majority of AGMs close to the 50S stage in the cloning experiment.

Enrichment for cells expressing the AA4.1 marker allowed recovery of the majority of the multipotent cells.

When the AA4.1<sup>−</sup> fraction was tested, no multipotent precursor was detected in 200 cells plated (data not shown). Importantly, all progenitors with lymphoid potential analyzed also had erythromyeloid potential, suggesting their multipotentiality. The rare macrophage colonies observed are likely to derive from blood contamination. The highest frequency of multipotent clones was found in 35–45S AGM when one out of four plated cells could differentiate in both a lymphocyte and a myeloid progeny. Considering that cell separation by panning also enriches for nonspecific adherent cells, the frequencies obtained could well represent one out of one hemopoietic progenitor differentiating in vitro. We then dissected the aorta and attempted to enrich hemopoietic cells using two different hemopoietic markers (Table II, bottom). AA4.1-expressing cells were highly enriched for multipotent progenitors, as previously shown for the whole AGM; in contrast, Sca-1 enrichment resulted in recovery of few multipotent progenitors, indicating either low levels or heterogeneous expression of this surface marker in the AGM population.

As shown above, most progenitors in the AGM were multipotent cells also capable of generating erythromyeloid progeny after in vitro expansion on stromal cells. However, it could be conceived that myeloid differentiation occurs in situ. To test this possibility, AGMs were dissociated upon explantation, and erythromyeloid colony assays were performed immediately. Circulating blood cells were used as positive control and cells dissociated from the developing limb buds as negative control. In this rudiment, we expected to detect only circulating precursors trapped in the blood vessels. The results displayed in Table III show the number of colonies per explant and/or blood obtained from one embryo. Committed erythroid progenitors such as CFU-E and BFU-E were fewer in the AGM than in the limb bud. However, the numbers of mixed colonies were similar to the number of B lymphoid progenitors previously found (compare Table III and Fig. 1) and consistently higher than in the limb bud, suggesting that they represent the progeny of the AGM multipotent cells.

*Development of the Omentum and Spleen.* The omentum rudiment becomes first identifiable at 10.5 dpc as a thick



**Figure 3.** Flow cytometry profile showing the lymphoid progeny of a single micromanipulated AGM cell. (Top) After culture in T lymphoid conditions. Cells of donor origin were gated as Ly5.1<sup>−</sup>. (Bottom) After culture in B lymphoid conditions. Lymphocytes were gated on forward and side scatter.

**Table III.** Quantification of Erythromyeloid Progenitors in the AGM

	AGM		Blood	Limb bud
	10.5 dpc	11.5 dpc	11.5 dpc	11.5 dpc
CFU-E	17	3	515	8
BFU-E	40	32	8,620	62
CFC-Mix	65	48	43	3

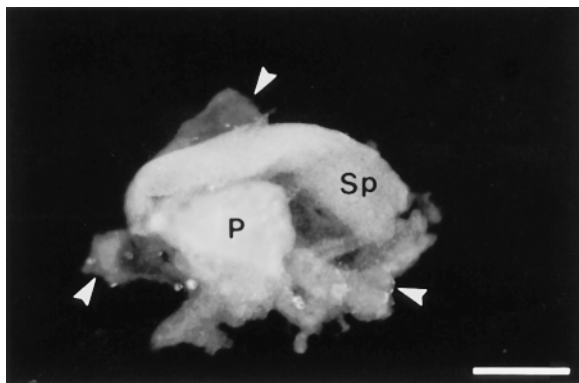
Cells from AGM, peripheral blood (positive control), and dissociated limb buds (negative control) were plated in methylcellulose under conditions that support multilineage erythromyeloid colony formation. CFU-E were counted on day 3, BFU-E and CFC-Mix on day 7 of culture.



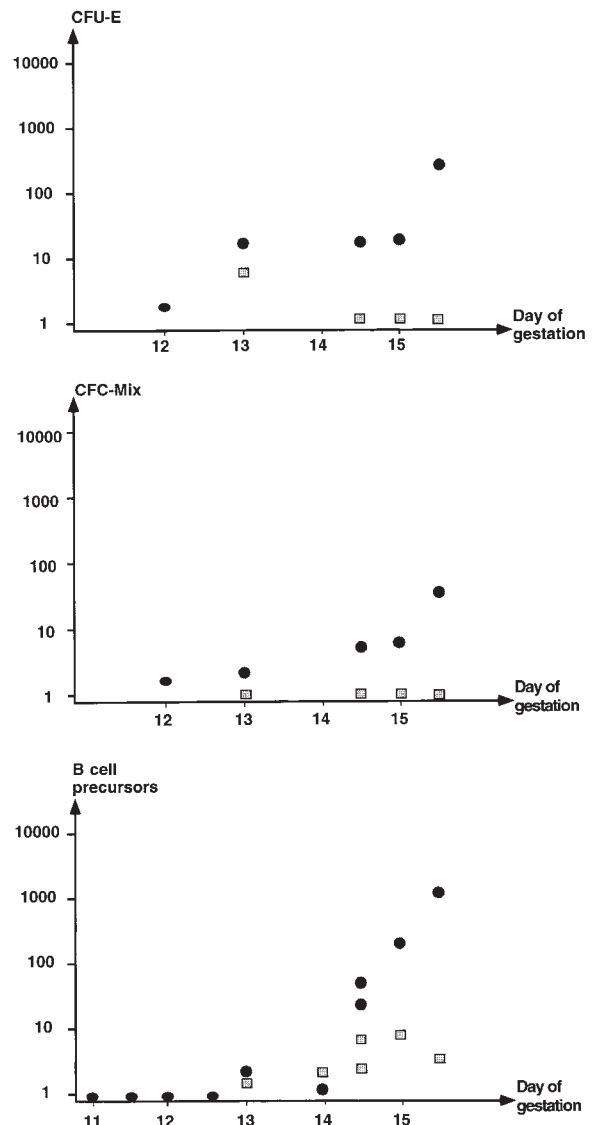
mesodermal layer covering the enlarged gut pocket that will give rise to the stomach. As development proceeds, this layer becomes thinner and vascularized. At 12 dpc, the spleen rudiment develops inside the omentum as two thickenings extending diagonally from the lower left and the upper right side to the middle of the stomach. The splenic rudiment is soon distinguishable from the remainder of the omentum by an increasing number of bright red spots, a sign of active erythrocyte accumulation. At 13.5 dpc, the rudiment has acquired the elongate shape typical of mouse (Fig. 4). Up to 12 dpc, the omental rudiment, including the developing spleen, was analyzed as a whole in our various experiments. As soon as the spleen rudiment could be accurately identified within the omentum, both rudiments were dissected and the progenitor potentials were assessed separately.

Lymphoid cells have been identified in the embryonic omentum at 14.5 dpc (15, 16). However, their origin and differentiation potential are as yet unknown. To investigate the ability of the omentum to generate hemopoietic cells, the early rudiments were cultured organotypically, before the quantification of progenitors. The differentiation potential was then assessed through in vitro colony assay and in vivo reconstitution of irradiated adults. This procedure was applied beginning at early developmental stages, from 11 to 15.5 dpc.

*Contrary to the Spleen, the Omentum, although Harboring Lymphoid Precursors, Does Not Carry Out Hemopoiesis.* B cell progenitors, CFU-E and CFC-Mix, were numbered between 11 and 15.5 dpc (five separate experiments; Fig. 5). At 11–12 dpc, when the spleen rudiment cannot be distinguished from the omentum, cells isolated from the omentum and spleen yielded a few myeloid colonies comparable to that from nonhemopoietic tissues (limb bud), likely trapped from the systemic circulation, with less than one B cell progenitor per omental and splenic rudiment. At 13 dpc, the omentum and spleen each yielded less than five progenitors endowed with B lymphoid potential. During the subsequent developmental stages, the frequency of these precu-



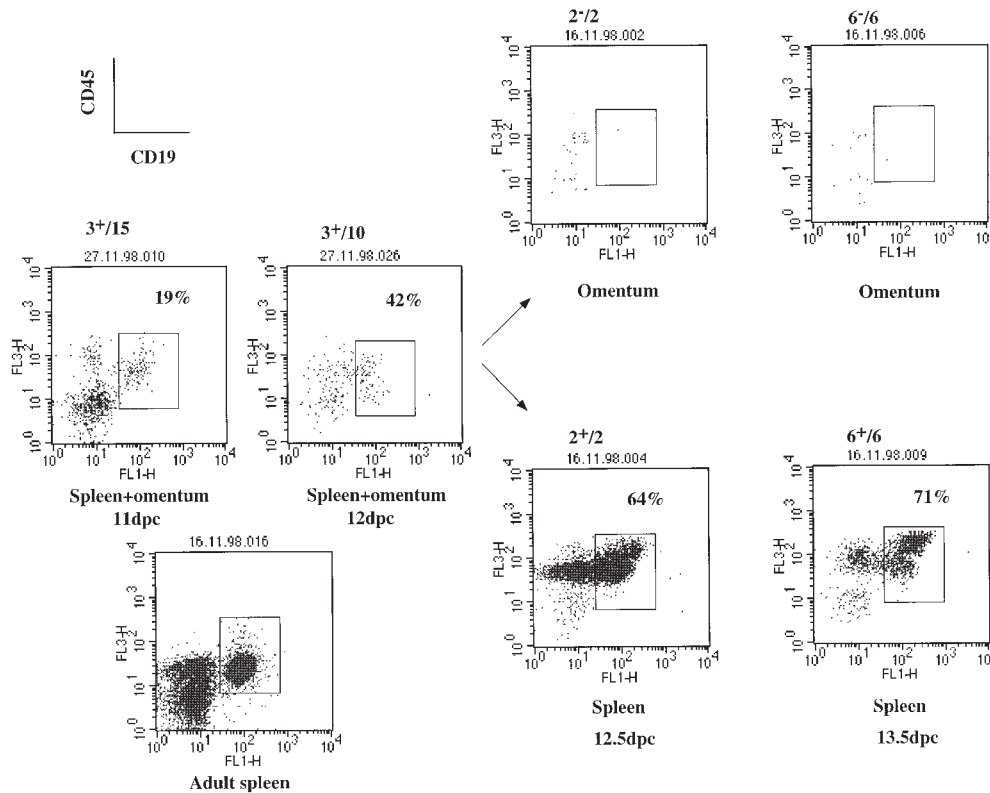
**Figure 4.** Anatomical relationship between omentum and spleen. The omentum and spleen explanted from a 14.5-dpc embryo are shown after being removed from the underlying stomach. The pancreas is then dissected out, and the two organs are separated (bar, 0.5 mm). Omentum, arrowheads; Sp, spleen; P, pancreas.



**Figure 5.** Quantification of erythromyeloid precursors CFU-E, CFC-Mix, and B lymphoid precursors in omentum and spleen over the mid-gestation period. Ovals, explant comprising both the omental and splenic rudiments (before 13 dpc); circles, splenic explant; squares, omental explant.

sors remained constant in the omentum (3–10 per organ), whereas in the spleen their number increased regularly and reached >1,000 progenitors per spleen at 15.5 dpc, the latest stage analyzed. In the case of myeloid precursors, whose frequency in the blood declines rapidly, the omentum that contained rare CFU-E and CFC-Mix at 12–13 dpc completely lacked all types of colony-forming progenitors thereafter, confirming that the rare myeloid colonies found at 11.5–12 dpc derived from precursors present in the circulation. In contrast, increasing numbers of progenitors were present in the spleen rudiment (Fig. 5).

When limiting numbers of 14–15-dpc omentum cells were allowed to repopulate irradiated fetal thymic lobes, a frequency of 1 out of 3,000 T cell precursors was found, representing 4 precursors per omentum (data not shown).



**Figure 6.** Flow cytometry profile showing CD19 (FL1) and Ly5 (FL3) staining of explanted omental-splenic rudiments (11 and 12 dpc), omenta, or spleens (12.5 and 13.5 dpc), after 12 d of organotypic culture. Lymphocytes were gated on forward and side scatter. The ratio of explants containing CD19<sup>+</sup> cells appears above the corresponding profile.

It has previously been stated that the murine splenic rudiment is colonized by 15 dpc (14). The results described above indicate that both omentum and spleen contain hemopoietic progenitors from 14.5 dpc. However, none of the experiments allowed us to discriminate whether the progenitors detected were generated in situ or were seeded from the circulation. Therefore, we introduced an organ culture step to discriminate between these two possibilities. As stated above, this culture step allows the emergence of HSCs in structures isolated from the embryos before this event normally takes place (4). Explants isolated from embryos at 11 and 12 dpc (38–50S) comprised both rudiments, whereas at 12.5 and 13.5 dpc the spleen and omentum were separated. Fig. 6 shows the flow cytometric profiles of cells dissociated from individual explants after 12 d in culture and stained with antibodies recognizing CD45 and CD19, a B lineage-specific marker. At 12.5 and 13.5 dpc, all splenic explants analyzed contained a large fraction of cells of the B lineage, whereas they were completely absent from the omentum explants. At earlier stages, 19 out of 25 explants analyzed were completely negative for CD19<sup>+</sup> cells, showing that hemopoietic cells observed later do not originate in situ. 6 out of 25 explants did show a low representation of CD19<sup>+</sup> cells, indicating that the process of colonization might already have started in a few cases.

We also tested the presence of LTR HSCs in the omentum and spleen, i.e., cells isolated from both organs were used to reconstitute the hemopoietic system of lethally irradiated mice, for >6 mo. 14.5-dpc embryos were chosen as donors, since in vitro quantification of B cell progenitors

showed that this is the earliest stage at which progenitors are present in significant numbers in both organs.

Omentum and spleen were explanted from 14.5-dpc embryos bearing the Ly5.2 allele of the CD45 antigen. Cells suspensions from two to four omenta, or one to three spleen equivalents, were injected intravenously per recipient into lethally irradiated Ly5.1 mice, together with  $5 \times 10^5$  syngeneic bone marrow cells (Ly5.1). After 6–8 mo, the contribution to hemopoiesis of donor-derived cells was analyzed by flow cytometry in the bone marrow, thymus, spleen, and PeC, and results are shown in Table IV.

None of the six mice grafted with omentum harbored donor-derived cells, indicating that undetectable numbers of hemopoietic stem cells are present in this organ. In contrast, 4 out of the 11 mice injected with embryonic spleen cells showed long-term multilineage reconstitution from donor-derived cells, albeit with low contribution in 2 cases.

Altogether, these results show that the splenic rudiment is colonized by 12.5 dpc and actively starts its hemopoietic activity around day 14.5 of gestation. At this stage, LTR activity is readily detected, suggesting that the spleen is colonized by stem cells. In contrast, the omentum harbors a limited number of committed lymphoid precursors that do not significantly expand with time.

## Discussion

Fetal liver and thymus are the major lymphohemopoietic organs during mouse embryonic development. The absence of hemopoiesis in organ cultures of both rudiments

**Table IV.** *LTR Assays*

No. of mice injected	Embryo equivalent per recipient	Percentage of reconstitution	Bone marrow		Thymus	Spleen	PeC
			Gr-1	B220	CD4 and/or CD8	B220	B220
4	1 spleen	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
2	1.5 spleens	62; 1.33	46; 1	76; 5	65; 1	75; 2	70; 5
1	2 spleens	3.3	1	3	1	8	4
2	2 spleens	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
1	3 spleens	75	57	78	90	78	79
1	3 spleens	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
5	2 omenta	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
1	4 omenta	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5

Ly5.1 mice were irradiated and injected with 14.5-dpc Ly5.2 spleen or omentum together with syngeneic bone marrow cells. Mice were analyzed 6–8 mo later. Shown are the percentages of donor-derived cells in the analyzed hemopoietic organs. The degree of reconstitution was measured after staining cells from different organs with Gr-1, B220, CD4 and CD8, and Ly5.2 mAbs.

(1–3), isolated early in ontogeny, showed that hemopoietic cell generation does not occur in situ. In addition, in the avian model, transplantation of early spleen and thymus rudiments showed absence of donor-derived progenitors and colonization by extrinsic progenitors (13, 24). Together, these results established that hemopoietic progenitors developing in the major hemopoietic organs are not generated in situ. This is also true of the bone marrow, the main hemopoietic organ in adult mammals, which is thought to be colonized by stem cells of fetal liver origin.

We and others have previously shown that intraembryonic candidate progenitors responsible for the establishment of definitive hemopoiesis are present in the mouse in the P-Sp at 8.5–10 dpc (4, 5) and in the AGM at 10.5–11 dpc (6, 7). Although AGM progenitors display LTR activity in adult recipients (6), those found in P-Sp are capable of reconstituting only newborn recipients after intraliver injection (25). These results indicate that either competitive reconstituting ability or homing properties of the intraembryonic progenitors differ at these two different stages. Arguments favoring a continuous generation of hemopoietic progenitors in the P-Sp and AGM are based on the fact that both sites represent the same anatomical structure at two distinct developmental stages. Moreover, intraembryonic hemogenic potential is, at these stages, restricted to these sites.

By quantifying in vitro progenitors in the AGM, we show here that the number of progenitors in the P-Sp/AGM increases regularly, suggesting that the process of generation and/or amplification of progenitors, as well as their release into the blood stream (8), is continuous. The in vitro behavior of AGM progenitors is identical to that of P-Sp progenitors (5). P-Sp/AGM progenitors are both enriched in populations expressing the AA4.1, rather than the Sca-1, antigen. More importantly, progenitors isolated from both sites remain multipotent, as shown by single cell fate analysis. The AGM is a site where no active hemopoiesis takes

place, as shown by the absence of lineage-restricted erythromyeloid colonies. We were consistently unable to detect committed erythromyeloid precursors above levels detected in our negative control, showing that the evolution to that stage does not occur within the AGM. Our unpublished results (Manaia, A., and I. Godin) point to a similar behavior in the P-Sp at earlier stages. Consistent with this result, single micromanipulated cells were capable of giving rise to erythroid, myeloid, and lymphoid progenitors when cultured on the stromal cell layer, reinforcing the notion that AGM is not a site where hemopoietic differentiation occurs. Thus, the population of hemopoietic progenitors that emerges in the AGM and reaches its maximum size at 11 dpc, before entering the circulation, constitutes a candidate for a pure stem cell pool.

We favor the view that hemopoietic progenitors in the P-Sp and AGM are the product of one single and continuous generation process. However, their capacity to reconstitute hemopoiesis in adult recipients changes with time. We hypothesize that the first multipotent cells released in circulation between 9 and 10 dpc do not yet have the full array of properties necessary to colonize hemopoietic organs (4, 6). As mentioned above, thymus and fetal liver are colonized starting at 10–11 dpc. At this stage, AGM progenitors are already capable of LTR activity in adult recipients (6), and might be the first colonizing cells in vivo.

Finally, the subdivision of the AGM into various components allowed us to allocate the progenitors to the aorta and surrounding region. This restriction to the aorta increases as development proceeds (55% at 30–35S, 62% at 35–40S, and 100% at 12–13 dpc). These experimental data may reflect improved accuracy in tissue separation, since the organ boundaries are better defined as development progresses. Alternatively, it might reflect an active displacement of cells into the lumen of the aorta from where they migrate to colonize the hemopoietic organs (8). Previous in situ analyses aimed at localizing intraembryonic hemogenic



sites already pointed to the aorta, as clusters of cells localized in the ventral wall of this vessel (Fig. 2 C) specifically express markers for early hemopoietic cells (26–28).

Based on the expression of  $\beta$ -gal in the mesonephros of *Ly6E.1-lacZ* transgenic mice, Miles et al. (29) concluded that the mesonephros was the major site of hemopoietic production in the AGM. Here, we find that the mesonephros is consistently devoid of hemopoietic progenitors. It is possible that Sca-1 was detected in cell types other than hemopoietic progenitors, since in the adult, Sca-1 is expressed in various nonhemopoietic cell types, including the kidney epithelium (30, 31). Moreover, cell separation experiments indicate that AGM progenitors are more efficiently enriched using the expression of the AA4.1 antigen than with Sca-1. As shown previously (32), the Sca-1 antigen is significantly expressed in the AGM and fetal liver only after 11 dpc. As reported previously (7), we found progenitors along the entire length of the AGM, but their number is higher in the anterior part.

To determine whether indeed *de novo* hemopoietic generation and hemopoietic differentiation are two incompatible properties always occurring in independent intraembryonic locations, we analyzed the omentum and spleen for the capacity to generate hemopoietic cells. We cultured the early rudiments *in vitro* in organ culture conditions, a method previously shown to permit the emergence of hemopoietic progenitors in a site that does not harbor hemopoietic progenitors at the time of explantation (4). Both organ culture and limiting dilution analysis indicate that, before 12.5 dpc, most omentum-splenic rudiments yielded no hemopoietic progeny, excluding an *in situ* generation of hemopoietic cells.

The spleen contains the first detectable lymphomyeloid progenitors at 12.5 dpc, consistent with previous findings in both mice (14) and chickens at an equivalent stage (13). We have previously shown that, before day 13 of gestation, all circulating progenitors with lymphoid potential are multipotent cells (8). Therefore, we conclude that the spleen is colonized by multipotent progenitors, originating in the P-Sp/AGM. This conclusion is confirmed by our results showing LTR activity in the spleen 1 d later. The exponen-

tial increase in colony-forming progenitors between days 14 and 15.5 of gestation points to 14.5 dpc as the beginning of hemopoietic activity in the spleen.

The omentum harbors a constantly low number of B and T cell precursors ( $\sim 10$ , from 13–15.5 dpc) and consistently lacks erythromyeloid CFCs throughout this period. In addition, we failed to detect LTR activity in the omentum rudiment even when cells pooled from four structures were injected into a single recipient. We conclude that this site is colonized by committed lymphoid progenitors, between 14 and 15 dpc, and that no expansion occurs *in situ*, as shown by both *in vitro* colony numeration and organotypic culture.

A picture emerges from this and previous studies indicating that hemopoietic progenitors can be obtained from 7.5-dpc organ-cultured intraembryonic splanchnopleura. The first progenitors that can differentiate into all hemopoietic lineages, when the intraembryonic splanchnopleura is immediately dissociated into single cells, are found at 8.5 dpc. These cells are very few and, although multipotent, have poor reconstituting activity. Thereafter, their numbers increase, and they progressively acquire LTR activity (10.5–11 dpc) when transplanted into an irradiated adult recipient, 1 d earlier than the fetal liver (11.5–12 dpc). The consistent absence of signs of hemopoietic differentiation in the P-Sp/AGM and, conversely, the incapacity of hemopoietic organs to generate *de novo* hemopoietic progenitors show that both activities are environmentally incompatible. Considering the timing of adult LTR activity as a landmark and the consecutive appearance of this activity in the AGM and fetal liver, it is reasonable to conclude that AGM progenitors home to the fetal liver. The extinction of hemopoietic cell generation in the AGM by 12.5 dpc and the failure to detect this activity elsewhere indicate that P-Sp/AGM progenitors are the only HSCs generated *de novo*. Although exact estimations are difficult, the total number of intraembryonic HSCs generated in the mouse should not exceed 500 cells, as calculated from results shown in Fig. 1. This initial pool of stem cells will further expand (self-renewal) in the primary hemopoietic organs throughout life.

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